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Induction of Heme Oxygenase-1 before Conditioning Results in Improved Survival and Reduced Graft-versus-Host Disease after Experimental Allogeneic Bone Marrow Transplantation

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ABSTRACT

Acute graft-versus-host disease (aGVHD) remains one of the main obstacles after allogeneic bone marrow transplantation (BMT). Using a well-established mouse BMT model in which aGVHD is induced across a haploidentical mismatch, we show that the expression of heme oxygenase-1 (HO-1) can be induced by cobalt-protoporphyrin IX (CoPP) in aGVHD target organs such as liver and bowel and that the induction of HO-1 before BMT results in improved overall survival and reduced aGVHD. Serum levels of proinflammatory cytokines were markedly reduced in CoPP-treated animals. Recipients displayed less damage to the intestinal mucosa, and this resulted in reduced serum lipopolysaccharide levels at day 6 after transplantation. Peritoneal cells and CD45$^+$ liver cells isolated from mice that received transplants strongly expressed HO-1 and displayed a reduction in the expression of activation markers such as CD11b, CD80, and major histocompatibility complex class I. This resulted in reduced T-cell activation ex vivo. These results demonstrate that the induction of HO-1 before high-dose conditioning protects the host in multiple ways and effectively ameliorates aGVHD.

KEY WORDS

GVHD • Cytokines • Heme oxygenase • BMT • LPS • Protoporphyrin • CoPP

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is an important therapeutic option for a number of malignant and nonmalignant hematopoietic disorders. Unfortunately, the utility of BMT is limited by several complications, including acute graft-versus-host disease (aGVHD), which remains the major cause of mortality after transplantation. The pathophysiology of aGVHD is believed to be a multistep process that involves conditioning-related toxicity, activation of donor T cells that recognize major histocompatibility complex (MHC), the presentation of alloantigens by host antigen-presenting cells (APC), and the release of proinflammatory cytokines [1,2]. Activation of donor T cells results in their proliferation and differentiation and eventually leads to tissue damage in aGVHD target organs such as liver, gut, and skin [3,4]. In addition to tissue damage by T cells, proinflammatory cytokines such as tumor necrosis factor (TNF)–α and interferon (IFN)–γ, released by various cells, including host APCs, contribute to the process [5-7]. This inflammatory response is propagated by the leakage of bacterial lipopolysaccharide (LPS) across bowel mucosa damaged initially by BMT conditioning and later by aGVHD; LPS is a potent cellular activator, and

LPS stimulation results in an increased proinflammatory cytokine release and an enhanced allostimulatory capacity of APCs.

The role of BMT conditioning in initiating the inflammatory events that contribute to the development of aGVHD and other transplant-related complications has prompted investigators to develop and test reduced-intensity preparative regimens. Unfortunately, several underlying diseases require full-intensity conditioning; thus, protection of the host against the injurious effects of radiochemotherapy could result in reduced GVHD.

The stress-inducible protein heme oxygenase-1 (HO-1), which catalyzes the rate-limiting step in heme degradation to biliverdin [8], has been identified as a protein with anti-inflammatory and antiapoptotic properties [9-12]. The enhanced expression of HO-1 results in local downregulation of inflammation and, in case of solid organ transplantation, leads to a prolonged graft survival [10,13-16]. Additionally, HO-1 has been implicated in the cytoprotective response against irradiation and apoptosis-inducing cytokines such as TNF-α [11,17,18] and is believed to mediate anti-inflammatory effects of interleukin (IL)–10 in mice [19]. We therefore hypothesized that enhanced expression of HO-1 before and after allogeneic BMT would abrogate the development of aGVHD. HO-1 can be induced in a variety of tissues by its natural substrate heme and other synthetic metal porphyrins, such as cobalt-protoporphyrin IX (CoPP) and zinc-protoporphyrin IX (ZnPP). Induction of HO-1 function is dependent on the central metal atom of the porphyrin, and in case of ZnPP, HO-1 function is blocked because the substrate cannot be enzymatically degraded [20,21]. We tested our hypothesis by using a well-described high-dose conditioning murine BMT model [22] in which aGVHD is characterized by inflammatory cytokine release and significant injury to the intestinal tract within the first week after BMT. Our results show that induction of HO-1 by CoPP in recipient mice before conditioning and BMT results in a reduction of GVHD and improved survival. GVHD reduction by HO-1 is mediated by a reduction in the production of proinflammatory cytokines, by injury to the gut, and by the allostimulatory capacity of host-derived APCs.

MATERIALS AND METHODS

Mice, BMT, and Assessment of GVHD

The protocol for BMT and GVHD induction has been described previously [23]. Briefly, female B6D2F1 (H-2^{bxd}), C57BL/6 (H-2^{b}), and CBA (H-2^{k}) mice were purchased from Charles River Laboratories (Sulzbach, Germany) and underwent transplantation between the ages of 10 and 20 weeks. B10.BR (H-2^{k}) donor mice were bred in our facility. Animals were housed according to German animal protection laws and protocols of the local committee. Bone marrow was harvested from the femurs and tibias of donor mice. Cell mixtures of erythrocyte-lysed 5×10^6 bone marrow cells were supplemented with 1×10^6 splenic cells from either syngeneic (B6D2F1, CBA) or allogeneic (C57BL/6, B10.BR) donors, resuspended in Leibovitz L-15 medium (Life Technologies, Grand Island, NY) and transplanted into B6D2F1 (CBA) recipients via tail vein infusion (0.25 mL total volume). Before transplantation, host mice received 14 Gy (or 11 Gy in case of CBA recipients) of total body irradiation (accelerator, 150 cGy/min) delivered in 2 fractions, separated by 3 hours, to reduce gastrointestinal toxicity. Mice were subsequently housed in sterilized microisolator cages and received normal chow and autoclaved hyperchlorinated water for the first 2 weeks after BMT and filtered water thereafter. For HO-1 induction, recipient mice received 125 μg of CoPP intraperitoneally at day −2 and −1 before conditioning (day 0) and at transplantation. Control mice received phosphate-buffered saline (PBS) alone.

The severity of GVHD was assessed by a previously described clinical scoring system, which incorporates 5 parameters: weight loss (recipient mice were ear-punched, and individual weights were obtained and recorded on day +1 and weekly thereafter), posture (hunching), activity, fur texture, and skin integrity [22,24]. Mice from coded cages were evaluated weekly, and changes were graded from 0 to 2 for each criterion. A clinical index was subsequently generated by summation of the 5 criteria scores (maximum, 10). Survival was assessed daily after transplantation. According to German animal protection laws and local protocols by the committee, animals showing a GVHD score greater than 6 were killed and added to Kaplan-Meier statistics the same day.

Induction of HO-1

CoPP or ZnPP (Frontier Scientific Porphyrin Products, Logan, UT) was prepared in dim light because of the light sensitivity of the compounds. CoPP was dissolved in sodium hydroxide, and the pH was subsequently adjusted to 7 by adding equal amounts of hydrochloric acid. The final pH of 7.4 was achieved by further dilution with PBS. Before injection, pH was confirmed by using a pH meter (WTW, Weilheim, Germany). In control experiments, animals that received CoPP or ZnPP (to induce HO-1 protein but block HO-1 function) did not show any signs of toxicity such as hunching or reduction in mobility, nor were any effects of CoPP/ZnPP observed in treated animals over several weeks.

Preparation of Peritoneal Macrophages and Culture

Mice were killed with CO_2, and the peritoneal cavity was flushed subsequently 3 times with 5 mL of RPMI 1640 media supplemented with 10% fetal calf serum (FCS) and antibiotics. Cells were washed twice, counted, and plated in round-bottomed 96-well plates (Corning Costar, Wiesbaden, Germany).

Preparation of $CD3^+$ Splenic T Cells

One milliliter of collagenase H (1 mg/mL) in RPMI 1640 standard medium was injected into spleens and incubated for 1 hour at 37°C. Spleens were then carefully dissolved between glass slides and filtered over a 70-μm cell strainer (Falcon, Le Pont de Claix, France). Cells were incubated in 10% PBS/FCS and incubated with biotinylated antibodies against B220, Ter119, Gr-1, and CD11b. Cells were washed and subsequently incubated with streptavidin microbeads (Miltenyi, Bergisch Gladbach, Germany) and sorted according to the manufacturer's protocol. The purity of the negative fraction was determined by flow cytometric analysis with phycoerythrin (PE)–conjugated or fluorescein isothiocyanate (FITC)–conjugated antibodies against CD3, CD4, and CD8.

Preparation of $CD45^+$ Liver Cells

Liver tissue was minced with scalpels and further dissolved by a short collagenase H (1 mg/mL) treatment at 37°C in standard RPMI 1640 medium for 30 minutes. Tissue was further dissolved by subsequent vigorous pipetting and filtered through a 70-μm cell strainer. The single-cell suspension was incubated with 10% PBS/FCS at 4°C for 30 minutes and subsequently incubated with anti-CD45 PE-conjugated antibody. Cells were isolated by using anti-PE microbeads according to the manufacturer's protocol (Miltenyi).

Cytokine and LPS Analysis

Serum and culture supernatant levels of cytokines were analyzed with commercially available enzyme-linked immunosorbent assay kits (Biosource, Nivelle, Belgium) according to the manufacturer's protocol. Serum samples were diluted 50%, and culture supernatants were used undiluted. LPS quantification was performed with the QCL1000 test kit (BioWhittaker, Walkersville, MD).

Antibodies Used

Rabbit anti-mouse HO-1 (SPA-895; StressGen, Victoria, Canada), anti–β-actin (A2066; Sigma, Chemicals, Taufkirchen, Germany), anti-rabbit immunoglobulin horseradish peroxidase antibody, anti-rabbit immunoglobulin FITC (DAKO, Hamburg, Germany), PE- or FITC-conjugated anti-mouse CD45 (clone 30.F11), MHC class I (clone AF6-88-5), CD80 (clone 16-10A1), CD86 (clone GL1), CD3 (clone 145-2c11), CD4 (clone RM4-7), CD8 (clone 53-6.7), biotinylated anti-mouse Gr-1 (clone RB6-8C5), CD45R/B220 (clone RA3-6B2), TER119 (clone TER119), and CD11b (clone M1/70) were obtained from Becton Dickinson (Heidelberg, Germany).

Western Blot Analysis

All reagents and chemicals were purchased from Roche (Mannheim, Germany) or Sigma. Cell pellets were washed in wash buffer (40 mmol/L Tris-HCl, 0.3 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid [pH 8], 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.5 mmol/L sodium orthovanadate, and 0.2 mmol/L sodium fluoride). After washing, cells were lysed in lysis buffer (wash buffer plus 1% Nonidet P-40 [Roche, Basel, Switzerland]) for 30 minutes at 4°C and were then centrifuged at 16000*g* for 15 minutes. An aliquot of the supernatant was used for determination of the protein concentration by the Biuret method. The protein solution was mixed 1:1 with 2× Laemmli buffer (20% glycerol, 4% sodium dodecyl sulfate, 10% β-mercaptoethanol, 0.02% bromphenol blue, and 1.25 mmol/L Tris, pH 6.8), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with equal amounts of protein. After separation at 130 V for 2 hours, proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Freiburg, Germany). To avoid unspecific protein binding, membranes were blocked with 5% dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 hour at room temperature, washed, and then incubated with anti–HO-1 antibody (SPA-895) and anti–β-actin antibody (A2066) in 5% dry milk/TBST overnight at 4°C. The blots were washed 3 times and incubated with anti-rabbit immunoglobulin horseradish peroxidase antibody (P0448; DAKO) in 5% dry milk/TBST for 1 hour at room temperature. Immunoreactive bands were developed by using an enhanced chemoluminescence kit (Amersham Pharmacia Biotech).

Immunohistochemistry

Tissue sections (5 μm) of frozen tissue or cytospins of isolated cells were fixed in 2% paraformaldehyde for 5 minutes at room temperature, washed in PBS, and incubated with 10% FCS and 5% swine serum for 30 minutes at room temperature. Slides were then incubated with anti–HO-1 antibody (1:200 dilution) for 60 minutes at room temperature. Staining was visualized by using FITC-labeled anti-rabbit immunoglobulin G (DAKO). Sections were embed-

ded in fluorescence mounting media (DAKO) and analyzed with a Carl Zeiss (Göttingen, Germany) Axioskope microscope and Zeiss Axiocam together with Axiovision software.

Mixed Lymphocyte Reaction

T cells were prepared by negative selection by using MiniMacs (Miltenyi) sorting columns, and purity was determined by subsequent flow cytometric analysis with directly labeled antibodies against CD3, CD4, and CD8. Purity was generally >85%. Equal numbers of T cells (200000 per well) were then plated in quadruplicate in 96-well plates (flat bottom) and stimulated with irradiated (30 Gy) $CD45^+$ liver cells (100000 per well) from allogeneic B6D2F1 donors that received intraperitoneal CoPP or PBS 24 hours before preparation. After 48 hours of re-stimulation, ^{3}H-thymidine was added to the culture, and proliferation activity was assessed by a microplate scintillation counter (Packard Canberra, Dreieich, Germany) after 24 hours. Data are expressed as the mean counts per minute per well. For positive control, syngeneic (C57BL/6-derived stimulators) stimulated T cells received concanavalin A at a concentration of 2.5 μg/mL 12 hours before ^{3}H-thymidine.

Flow Cytometric Analysis

Cells were washed in freshly prepared cold PBS/5% FCS and incubated with 10% supernatant from clone 2.4G2 to block Fcγ receptors for 30 minutes on ice. Then, directly FITC- or PE-labeled monoclonal antibodies (H-2^k, I-A, CD80, CD86, CD4, CD8, CD3, CD11b, and CD11c) were applied and incubated for 30 minutes on ice. Cells were washed twice in cold PBS/FCS and subsequently analyzed by FACScan (Becton Dickinson).

Tissue Procurement and Examination of Histology

Mice were killed by CO_2, and organs were removed and placed into OCT Tissue Tek (Miles, Elkhart, IN) and snap-frozen in dry ice–cooled butanol. Organs were stored at −80°C. In some experiments, organs were fixed in 10% buffered formalin, embedded in paraffin, and cut into 5-μm-thick sections. Sections were stained with hematoxylin and eosin according to standard practice.

Coded small-bowel sections were scored by T.S. The scoring system includes 4 parameters from 0 to 2 (apoptosis, number of villi, number of Paneth cells, and number of mitoses per high-power field), giving rise to maximum score of 8.

Statistical Considerations

All values are expressed as the mean ± SEM. Statistical comparisons between groups were conducted with the nonparametric unpaired Mann-Whitney test unless otherwise specified. For survival curves, Kaplan-Meier estimates were used.

RESULTS

GVHD Target Organs Express HO-1 after the Administration of CoPP

Several studies have demonstrated that the expression of HO-1 can be induced in a variety of tissues, such as endothelium, lung, and kidney [10,12,14,16,25]. We first determined whether the administration of CoPP could enhance the tissue expression of HO-1 in GVHD target organs. B6D2F1 animals were injected intraperitoneally with 125 μg of CoPP on 2 consecutive days. Sections of liver and intestine were analyzed for HO-1 expression by immunohistochemistry 48 hours after the first injection, as described in Materials and Methods. In these experiments, splenic tissue served as a positive control because HO-1 is constitutively expressed in the spleen because of the natural abundance of its substrate heme (Figure 1A, left panel). HO-1 was expressed predominantly within the red pulp where activated macrophages reside. As shown in the middle panel of Figure 1A, endogenous expression of HO-1 was not observed in liver or small-bowel tissue after treatment with PBS. In contrast, HO-1 was readily apparent in cells residing between hepatocytes after the injection of CoPP; HO-1 expression was evenly distributed and surrounded central veins. Increased expression of HO-1 was confirmed by Western blotting with liver cell extracts (Figure 1A). Similar expression patterns were observed in the small bowel, where the distribution of HO-expressing cells was more patchy and organized in discrete groups of cells (Figure 1A, right panel).

The Administration of CoPP Significantly Reduces the Severity of aGVHD

We next determined the effects of enhanced HO-1 expression after CoPP administration on the severity of aGVHD after allogeneic BMT. B6D2F1 mice received BMT from either allogeneic (B6) or syngeneic (B6D2F1) donors after 1400 cGy of TBI. Animals from each group were injected with either CoPP or control (PBS) on days −2 and −1, as described previously. As expected, all syngeneic BMT recipients with or without CoPP survived (Figure 2A) and, after transient weight loss after TBI, showed no signs of GVHD (Figure 2B). In contrast, control-treated allogeneic BMT recipients died rapidly from aGVHD between days 7 and 21, and mortality was 100% by day 56 after BMT (Figure 2A). The administration of CoPP resulted in nearly complete abrogation of aGVHD mortality; approximately 90% of mice in the allogeneic group survived at day 63. Fur-

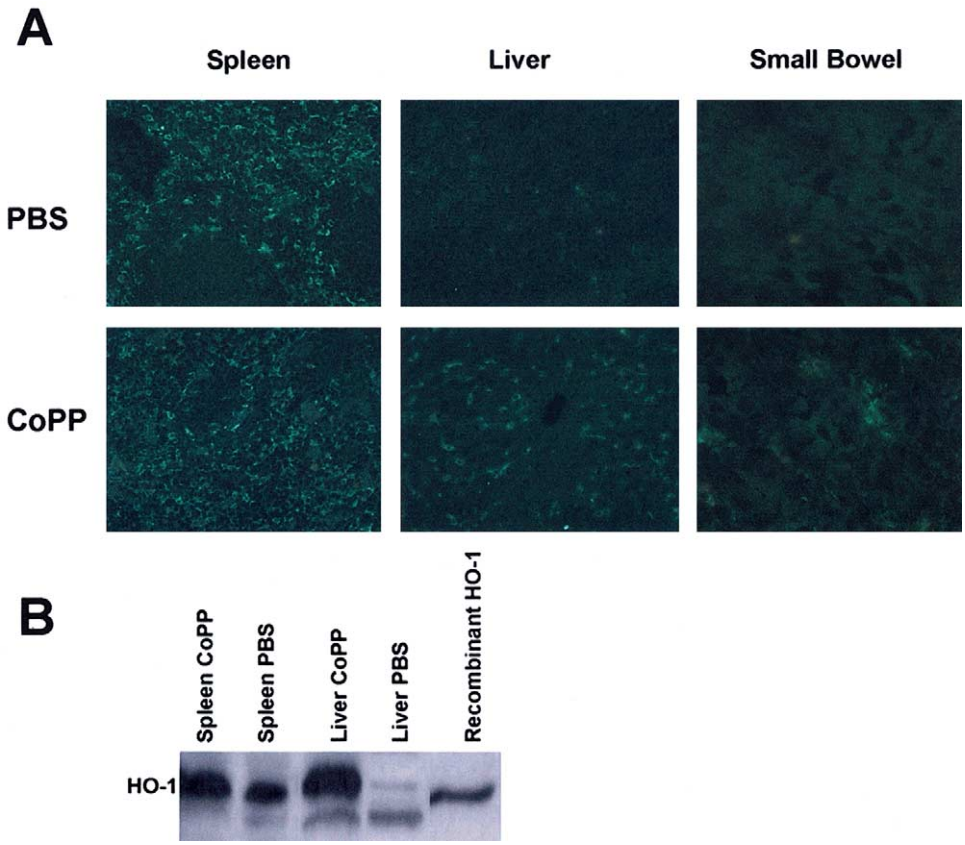

Figure 1. Induction of HO-1 in GVHD target organs and macrophages. A, The administration of CoPP increased HO-1 expression in GVHD target tissue. Naive mice were injected twice intraperitoneally with 125 μg of CoPP or PBS. Organs were harvested 48 hours after the first injection and evaluated for HO-1 expression, as indicated by the green fluorescence. Original magnification: spleen, 400×; liver and bowel, 630×. B, Western blotting of cellular extracts from liver or spleen samples confirmed in situ HO-1 staining.

ther evaluation revealed that the clinical scores of mice treated with CoPP were also significantly reduced on day 14 and day 21 ($P < .05$) compared with controls. Surviving mice in this group did, however, develop signs of GVHD over time, mainly reflected by alterations in skin integrity and fur texture, although the severity of aGVHD remained stable through day 63. Despite signs of GVHD, no additional deaths occurred. This underscores the significance of preventing early toxicity from GVHD. As an additional control, some animals from each group were treated with ZnPP, a substrate that induces HO-1 protein expression but blocks HO-1 function, and no differences in aGVHD were noted when compared with PBS-treated animals. This underscores the role of enhanced HO-1 function in reducing GVHD (data not shown). In a different model of aGVHD (B10.BR into CBA) in which GVHD is induced across multiple minor mismatches, similar results were observed. Animals received lower conditioning with 11 Gy of total body irradiation and underwent transplantation as described previously. When treated with CoPP before transplantation, 61% of the mice survived at day 42, as compared with 16% survival in the PBS-treated control group. GVHD scoring was also reduced on day 28 from 3.9 ± 0.5 to 2.0 ± 0.5 ($P < .08$) in the CoPP-treated group. In contrast to the haploidentical BMT model, early mortality between day 7 and 14 was similar in both groups. However, PBS-treated animals showed progressive GVHD and, therefore, higher mortality, whereas CoPP-treated animals remained stable, with a low GVHD score of approximately 2.

Administration of CoPP Significantly Reduces Serum Levels of Proinflammatory Cytokines after Allogeneic BMT

Previous studies using this BMT model have demonstrated a critical role for proinflammatory cytokine release in the development of aGVHD [22,23,26]. We therefore measured serum levels of TNF-α, IL-1β, IFN-γ, and IL-12 between day 4 and 6 after BMT, a time point that heralds the onset of mortality from GVHD in this system. As shown in Figure 3, serum TNF-α (48.6 ± 5.0 pg/mL versus 26.9 ± 3.8 pg/mL; $P < .002$) and IL-12 (26.3 ± 4.6 pg/mL versus 3.0 ± 0.7 pg/mL; $P < .002$) levels were markedly reduced in CoPP-treated animals compared with allogeneic controls. IFN-γ (623 ± 287 pg/mL versus 204 ± 61 pg/mL; $P < .13$)

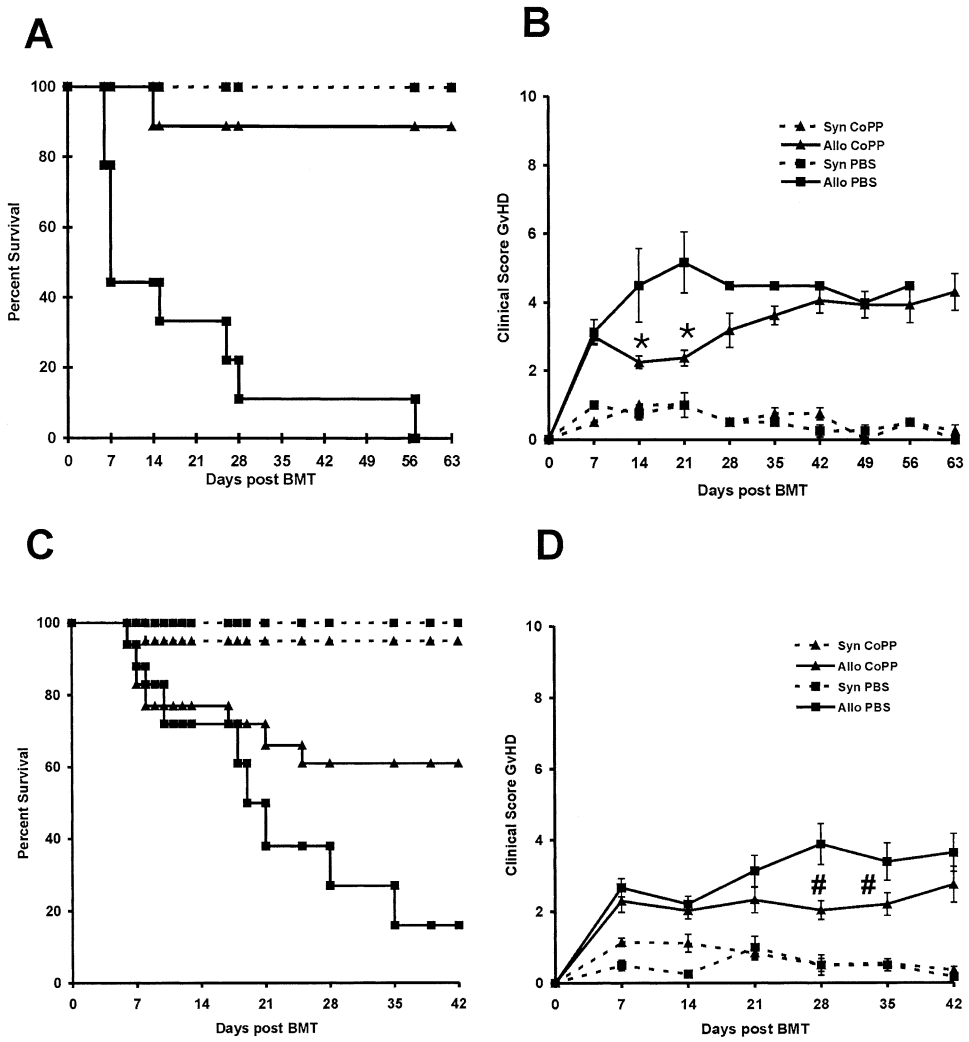

Figure 2. Reduction of GVHD and improved survival after transplantation in CoPP-treated animals. B6D2F1 mice were treated with CoPP or PBS on days −2 and −1 before 14 Gy of irradiation and BMT from either allogeneic (C57BL/6) or syngeneic (B6D2F1) donors on day 0. Mice were then monitored daily for survival and weekly for GVHD score as described in Materials and Methods. A, Mortality after allogeneic BMT was also markedly improved in mice treated with CoPP (triangles; solid line) compared with controls treated with PBS (square box; solid line; $P < .01$; Kaplan-Meier analysis). Syngeneic BMT recipients that received CoPP or PBS all survived. B, Treatment of B6D2F1 mice with 2 doses of CoPP (triangles; solid line) before conditioning resulted in a significant reduction of GVHD score between day 14 (*P = .038) and day 21 (*P = .024) when compared with control-treated animals (square box; solid line). The graphs (A and B) show representative results from 1 of 5 independent experiments. CBA mice were treated with CoPP or PBS on days −2 and −1 before 11 Gy of irradiation and BMT from either allogeneic (B10.BR) or syngeneic (CBA) donors on day 0. C, Mortality after allogeneic BMT (CoPP-treated mice, triangle; PBS-treated mice, solid square; $P < .01$; Kaplan-Meier analysis). D, GVHD score after allogeneic BMT (#$P < .08$). The graphs (C and D) show combined data from 3 independent experiments (12-18 animals per group).

and IL-1β (14.1 ± 3.9 pg/mL versus 7.0 ± 2.3 pg/mL; $P = .13$) levels were also reduced but did not reach statistical significance at the 5% level. However, serum IL-10 levels were significantly increased in allogeneic BMT recipients treated with CoPP compared with controls (39.9 ± 5.2 pg/mL versus 19.1 ± 4.5 pg/mL; $P < .05$). Consistent with previous work in this system, serum cytokine levels in syngeneic controls were below the limit of detection for the enzyme-linked immunosorbent assay kits used for this study (data not shown).

Administration of CoPP Reduces Macrophage Activation and Allostimulatory Capacity

Macrophages are the principal source of inflammatory cytokine production after allogeneic BMT and are also important APCs. We next evaluated the expression of HO-1 and activation markers on macrophages obtained from the peritoneal cavity and liver (ie, Kupffer cells) of animals that had undergone transplantation. On day 4 after BMT, peritoneal cells harvested from allogeneic BMT recipients treated

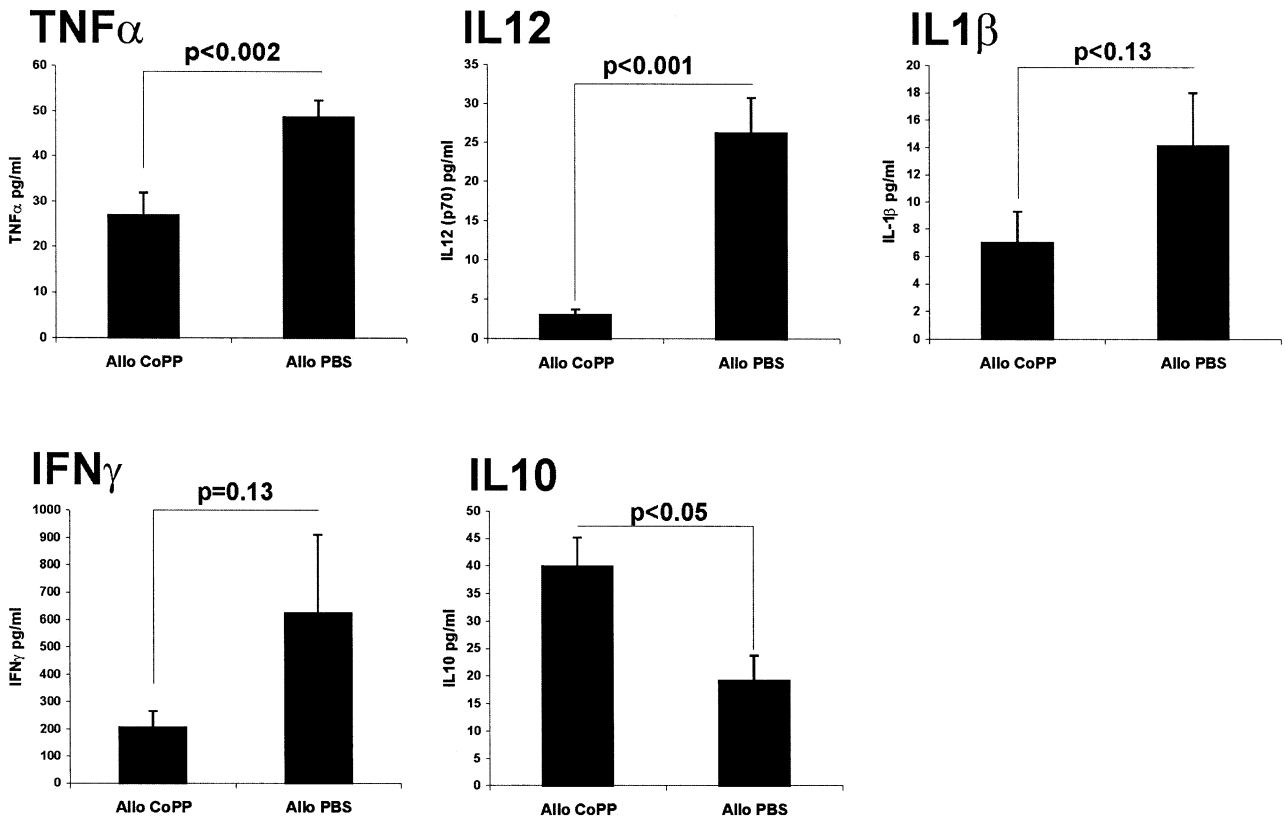

Figure 3. Proinflammatory serum cytokines were reduced in CoPP-treated animals. B6D2F1 mice were treated with either CoPP or PBS and received BMT as described in Figure 2. Serum cytokines were analyzed between day 4 and 6 after transplantation by enzyme-linked immunosorbent assay according to the manufacturer's protocol. Data are expressed as mean ± SEM (8 to 20 animals per group pooled from 3 independent experiments).

with CoPP readily expressed HO-1 (Figure 4A), as shown by immunohistochemistry and Western blot. Morphologically, most of these cells were macrophages (data not shown), and nearly 100% of peritoneal cells obtained from allogeneic control mice expressed CD11b (Figure 4B). Surprisingly, we observed a significant reduction in CD11b expression in CoPP-treated BMT mice. Because CD11b is also considered an activation marker, we next looked at the expression of MHC class I and the co-stimulatory molecules CD80 and CD86 and observed similar reductions in cell-surface expression (Figure 4B). Comparable reductions in the expression of these activation markers were also noted in the CoPP group when $CD45^+$, HO-1–expressing cells from liver tissue were isolated at day 6 after BMT (data not shown). We next determined whether the administration of CoPP altered the allostimulatory capacity of host APCs. $CD45^+$ liver cells were obtained from CoPP-, ZnPP-, or PBS-treated naive B6D2F1 mice (Figure 4D) and used to re-stimulate splenic T cells collected on day 6 from allogeneic BMT recipients. Liver cells (rather than peritoneal cells) were chosen as stimulators to control for any direct toxic effect CoPP or ZnPP might have on cells resident to the peritoneal cavity. As shown in Figure 4C, T-cell proliferation was reduced by 50% when the stimulator cells were obtained from CoPP-treated mice compared with control or ZnPP-treated mice. However, the decrease in proliferative response ex vivo was not associated with reduced IFN-γ or increased IL-10 production in culture (data not shown).

CoPP Treatment Results in Lower Serum LPS Levels and Preservation of Small-Bowel Mucosa

The intestinal tract has been shown to be particularly sensitive to the injurious effects of inflammatory cytokines released during the induction of aGVHD [22,26,27]. We hypothesized that the reduction in TNF-α levels would therefore be associated with a reduction in the severity of gut GVHD. Samples of small bowel were harvested on day 6 after BMT and analyzed microscopically to generate an index of injury as described in Materials and Methods. As shown in Figure 5A, small-bowel sections from syngeneic BMT recipients (left panel) demonstrated re-establishment of near-normal intestinal architecture, whereas sections from allogeneic BMT mice treated with PBS demonstrated severe signs of tissue damage. These changes included crypt destruction and drop-

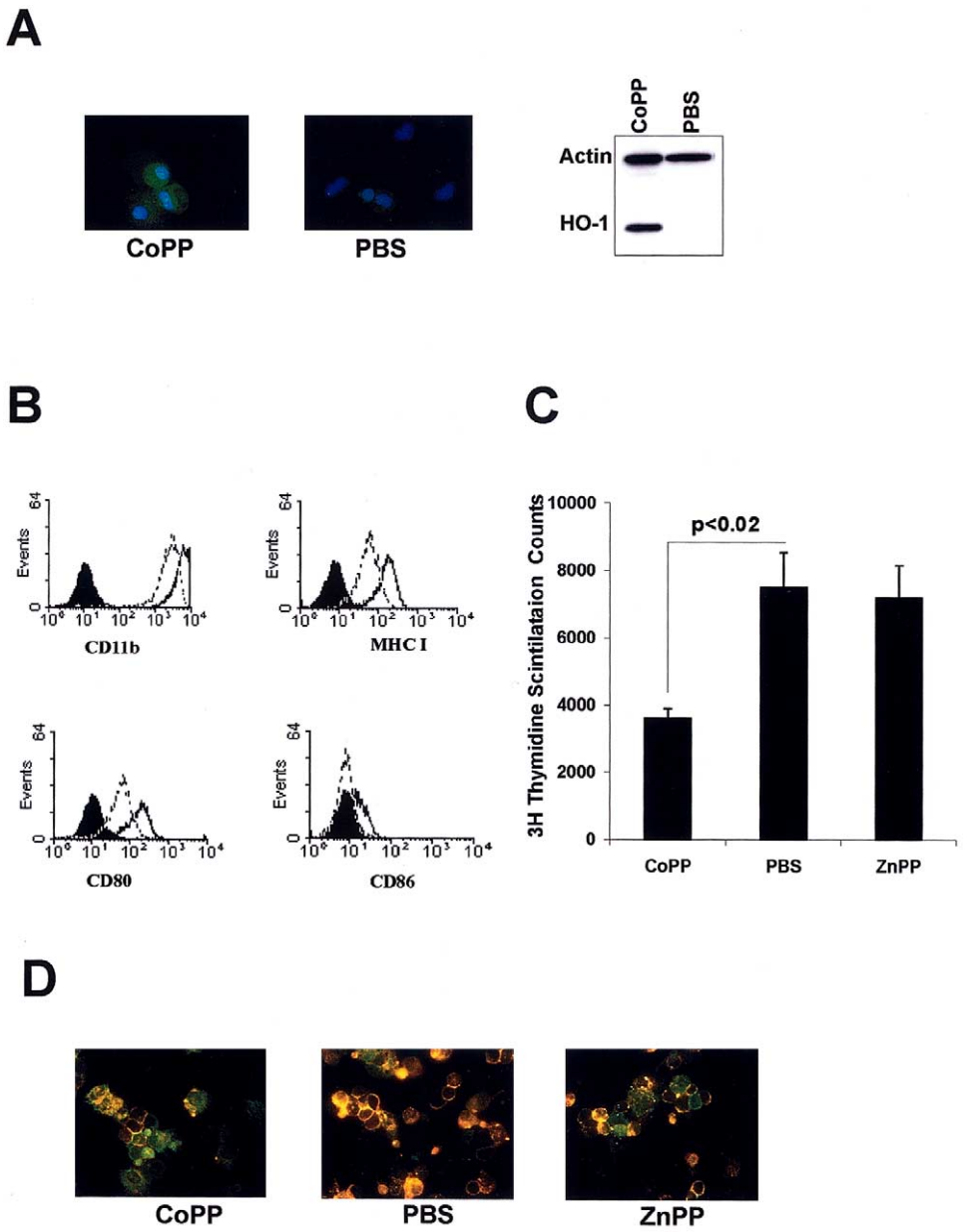

Figure 4. Peritoneal macrophages displayed reduced surface expression of activation markers and failed to stimulate allogeneic T cells. A, B6D2F1 mice were treated with CoPP or PBS on days −2 and −1 before lethal irradiation and allogeneic BMT from C57BL/6 donors on day 0. Peritoneal cells were harvested and stained for HO-1 expression as described in Materials and Methods. Macrophage expression of HO-1 was enhanced in BMT recipients treated with CoPP, but not with PBS. Original magnification, 630×. Western blotting of cellular extract antibody from macrophages treated with CoPP revealed a strong HO-1 induction. B, Peritoneal cells were isolated on day 6 after BMT, as described previously, and were analyzed by flow cytometry for surface expression of CD11b, MHC class I, CD80, and CD86. The solid line indicates cells from PBS-treated animals, and dotted lines represent CoPP-treated recipients. Cells from CoPP-treated mice displayed a reduced surface expression of macrophage markers such as CD11b. In addition, MHC class I expression and expression of co-stimulatory molecules such as CD80 were reduced. Filled histograms represent the isotype control antibody (representative results from 1 of 3 experiments). C, Freshly isolated $CD45^+$ liver cells were used to re-stimulate allogeneic splenic T cells harvested at day 6 after BMT. Cells from PBS- or ZnPP-treated mice induced a greater T-cell proliferative response compared with stimulator cells obtained from CoPP-treated animals ($P < .02$). D, $CD45^+$ cells isolated from liver 48 hours after the induction of HO-1 with CoPP, ZnPP, or PBS. Cells from animals treated with CoPP or ZnPP displayed strong HO-1 expression, as indicated by the green fluorescence. Orange surface staining represents CD45 expression on isolated cells. Original magnification, 630×.

out, villous blunting, and surface erosion (middle panel). By contrast, treatment with CoPP resulted in preservation of small-bowel architecture, and only mild crypt destruction and villous blunting was observed in these animals (right panel). When tissue samples were examined in a coded fashion (T.S.) by

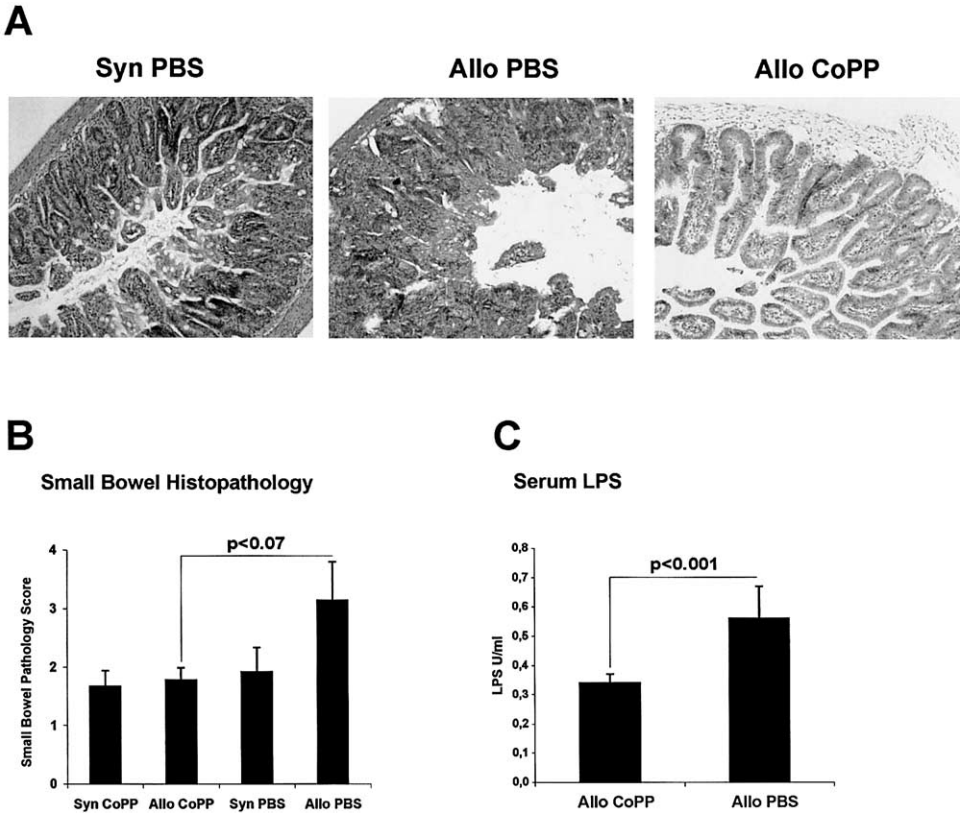

Figure 5. Treatment with CoPP reduced intestinal GVHD and serum LPS levels after allogeneic BMT. B6D2F1 mice were treated with either CoPP or PBS and received BMT as described in Figure 2. A, Small-bowel samples were harvested on day 6 after BMT and prepared for microscopic analysis as described in Materials and Methods. Small-bowel architecture was preserved in CoPP-treated allogeneic mice that received transplants compared with controls, as indicated by less villous blunting and crypt destruction. B, Microscopic features were incorporated into a semiquantitative scoring system to generate an index of injury. Mean small-bowel pathology scores were significantly lower in CoPP-treated animals compared with allogeneic BMT recipients treated with PBS. (Data are expressed as mean ± SEM; Mann-Whitney test; 8 to 12 animals per group). C, Serum samples were collected between days 4 and 6 after BMT and were analyzed for LPS by using the Limulus QL1000 test (BioWhittaker). Animals treated with CoPP before conditioning displayed significantly reduced serum LPS levels (data are expressed as mean ± SEM; Mann-Whitney test; 10 to 18 animals per group from 3 independent experiments).

using a scoring system that includes 4 parameters as signs of tissue alteration, pathology scores in CoPP-treated animals were decreased compared with allogeneic controls (3.14 ± 0.65 to 1.76 ± 0.2; $P = .07$) and were reduced to the level observed after syngeneic BMT (Figure 5B). Endogenous LPS has been shown to be an important trigger for inflammatory cytokine release after allogeneic BMT [28,29], and we measured serum LPS levels at day 4 to 6 after BMT. As shown in Figure 5C, the protective effect that CoPP administration had on the intestinal tract was associated with a reduction in serum LPS levels in these animals compared with allogeneic controls (0.34 ± 0.03 versus 0.56 ± 0.11; $P < .001$).

DISCUSSION

In this study we examined the effects of HO-1 induction on the development of aGVHD by using a well-established mouse BMT model. HO-1 expression could be demonstrated in GVHD target tissue, including the liver and gut, within 48 hours of CoPP injection. Induction of HO-1 before conditioning resulted in a reduction in the severity of aGVHD, as measured by mortality and clinical score. This protective effect was associated with significant reductions in inflammatory cytokine release, intestinal histopathology, and serum LPS levels. Additionally, the induction of HO-1 after BMT was associated with a reduction in the cell-surface expression of activation markers and in the allostimulatory capacity of peritoneal and hepatic macrophages.

Our results demonstrate that the induction of HO-1 before conditioning with 1400 cGy of TBI has a significant effect on overall survival and aGVHD after allogeneic BMT. The reduction of aGVHD in our model may be attributed to interactions with several aspects of GVHD pathophysiology. As described by Hill et al. [22,23], the development of aGVHD is believed to be a multistep process initiated by diffuse tissue injury caused by BMT conditioning regimens. Several studies in humans and in mice have revealed

the connection between high doses of irradiation and the development of aGVHD, and these have led to the development of reduced-intensity conditioning regimens to prevent damage to the host and the concurrent release of proinflammatory cytokines [3,22,30,31]. Only a few studies have been completed to reduce conditioning-induced damage by protecting the host tissue [32,33]. Our data suggest that the effects of HO-1 induction are related in part to the protection of BMT recipient mice against damage from TBI, particularly with respect to radiation-sensitive organs such as the small bowel; reduced intestinal injury was associated with significant reductions in serum LPS levels. Several studies have demonstrated that the induction of HO-1 can prevent irradiation-induced programmed cell death [10,12,17]. In particular, HO-1 induction in endothelial cells protects the endothelium from various stress signals, including irradiation [34-37]. Endothelial cell damage, in particular, has been suggested recently as the primary lesion during the initiation of gut damage after irradiation [38].

The transplantation model used in these experiments is characterized by a strong release of proinflammatory cytokines such as TNF-α, IL-1β, and IFN-γ, which are significant contributors to GVHD target organ injury [2,22,26,39]. The induction of HO-1 by CoPP before conditioning significantly reduced serum levels of TNF-α and IL-12 and effectively prevented the early mortality seen in this system. In addition, the administration of CoPP before BMT was associated with a significant increase in IL-10 levels. Polarization of T cells toward a T-helper type 2 phenotype has resulted in improved survival after allogeneic BMT in several studies [23,40-42], but it remains to be determined in our studies whether the increased serum levels of IL-10 are a result of a shift in cytokine release by macrophages, T-cells, or both. The role of IL-10 after allogeneic BMT has been a subject of considerable interest. IL-10 is known to have "tolerogenic" effects on T cells when present in a mixed lymphocyte reaction together with monocytes [43-45]; it downregulates MHC class I and II expression on APCs and effectively suppresses T-cell proliferation and IFN-γ production. However, several groups have shown that the administration of IL-10 before and after allogeneic BMT can result in mixed outcomes [7,42,46-49]. In a study pertinent to our work, Lee and Chau [19] recently demonstrated that the anti-inflammatory effects of IL-10 in mice are mediated at least in part by HO-1. They demonstrated that HO-1 was induced by IL-10 and prevented macrophages from releasing TNF-α in response to LPS stimulation. The reduction in serum TNF-α levels in allogeneic BMT recipients treated with CoPP is in accord with this study.

In a previous publication, the effect of HO-1 induction on GVHD was explored by inducing HO-1 in the donor rather than the recipient [50]. Those authors used the same mouse strain combination, but it is important to note that BMT recipients did not receive any conditioning and that large doses of splenocytes were used to induce GVHD. The authors reported a decrease in the spontaneous release of IFN-γ and IL-2 and an increase in the spontaneous release of IL-10 when splenocytes harvested after BMT where cultured in vitro. Although these data suggest that HO-1 induction in BMT donors can polarize T cells toward a T-helper type 2 phenotype, the authors were not able to link improved survival or histologic changes in GVHD target organs to alterations of cytokine production in vivo. In contrast to the observations of Woo et al. [50], we did not observe any changes in ex vivo cytokine profiles despite the reduction of the proliferative response when macrophages from CoPP-treated animals were used as stimulators in a mixed lymphocyte reaction. In addition, we did not observe any differences in spleen size or splenocyte numbers after BMT. This shows that the administration of CoPP to BMT recipients reduced GVHD without significantly impairing donor T-cell expansion after transplantation. This point is exemplified by our observations that aGVHD was not completely abolished by the administration of CoPP; surviving mice displayed signs of aGVHD over the entire time of observation, as indicated by our GVHD scoring system. These findings have important ramifications with respect to whether the reduction in GVHD seen after CoPP administration will be associated with preservation of the beneficial graft-versus-leukemia effect, and experiments are planned to address this issue.

Our data demonstrate that 2 injections of CoPP to BMT recipients before the infusion of bone marrow and T cells result in nearly complete abrogation of GVHD mortality. This protective effect is associated with significant reductions in serum cytokine levels and injury to the intestinal tract but does not affect donor T-cell responses after BMT. These findings suggest that the induction of HO-1 expression before full-intensity conditioning may represent a novel, non–cross-reactive strategy to reduce the severity of GVHD and improve outcomes after allogeneic BMT.

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